AWARD NUMBER: W81XWH-13-1-0173

TITLE: Real-Time Visualization and Manipulation of the Metastatic Trajectory of Breast Cancer Cells

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REPORT DATE: September 2017

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
September 2017	Final	1Jul2013 - 30Jun2017

4. TITLE AND SUBTITLE
Real-Time Visualization and Manipulation of the Metastatic Trajectory of Breast Cancer Cell

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-13-1-0173

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Pittsburgh
Pittsburgh PA 15213

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The purpose of this work was to engineer breast cancer cells to irreversibly alter the genome of nearby cells through exosomal transfer of Cre recombinase from the cancer cells to surrounding cells. Our goal was to use this study to activate green fluorescent protein in the host reporter cells in the environment of cancer cells and simultaneously to delete PTEN from those host cells. We hypothesized that PTEN gene deletion in adjacent normal cells would increase the proliferation of low-aggressive or dormant cancer cells. In the first reporting period, we concentrated on optimizing Cre-containing constructs for exosomal packaging. We also initiated breeding and characterization of the mouse model for in vivo experiments. In the second reporting period, we generated in a doxycycline-controlled inducible, established stable D2.OR cell lines with inducible Cre that is functional in mice, and demonstrated robust recombination of the genome of host bystander cells by Cre-exosome producing D2.OR breast cancer cells that were injected subcutaneously. During the current reporting period, we bred floxed PTEN, Zsgreen transgenic mice to homogenousity, and commenced experiments to determine if Cre-exosome expression and resulting host stromal recombination enabled D2.OR cells to escape dormancy. Because D2.OR did not grow out under these conditions, we extended our study, as planned, to other Balb/C syngeneic cancer cell lines, 4T1 and D2.A1. These lines were rendered capable of exosomal Cre production and transfer under doxycycline control, and in vivo experiments are in progress.

15. SUBJECT TERMS

Nothing listed

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U Unclassified	U Unclassified	U Unclassified	UU	20	

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1. INTRODUCTION

The purpose of this work was to engineer breast cancer cells to irreversibly alter the genome of nearby cells through exosomal transfer of Cre recombinase (hereafter "Cre") from the cancer cells to surrounding cells. Our goal was to use this study to activate green fluorescent protein in the host reporter cells in the environment of cancer cells and simultaneously to delete PTEN from those host cells. The ability to induce desired host genetic changes in a topologically-restricted fashion by cell-cell Cre transfer was conceptually and operationally novel. We tested whether cancer cells could be generated in which Cre recombinase was fused to exosome delivery domains and that functional exosomal Cre fusion protein would be transferred to neighboring cells *in vitro* and *in vivo.* We further hypothesized that Cre-mediated loss of PTEN specifically in cells bordering quiescent breast cancer cells results in the activation and proliferation of those cancer cells through a paracrine mechanism

2. KEYWORDS

Exosomes
Breast cancer
Dormancy
Phosphatase and tensin homolog (PTEN)

3. OVERALL PROJECT SUMMARY

This project developed a technology to enable manipulated cells to cause DNA recombination within the genome of neighboring cells in their "exosomal space" i.e., neighboring cells that took up exosomal microvesicles secreted by the manipulated cells. We did this by arranging the packaging and transfer of Cre recombinase within exosomes produced and secreted by cells of interest, in this case, cancer cells. We were interested in demonstrating that cancer cells manipulated to express Cre in exosomes would recombine neighboring genomes and followed this by deletion of a stop codon in front of a green fluorescent protein in those genomes (leading to green fluorescence). In order to use this tool to study a critical biomedical problem, we focused initially on cancer dormancy. Several microenvironmental changes had been shown to effect the growth of cancer cells, and we were intrigued by a report that loss of the tumor suppressor gene PTEN in normal cells enabled more vigorous growth of breast cancer cells in a mouse MMTV-ErbB2 transgenic cancer model [1].

We hypothesized that PTEN deletion in stroma could accelerate the growth of slow growing or dormant syngeneic cancer cells and that this could be engineered by Cre- transfer from such cancer cells to adjacent stroma containing floxed PTEN. We therefore generated a double transgenic mouse that had both Cre-inducible ZsGreen fluorescence and Cre- deletion of floxed PTEN and back crossed it 7 generations to the Balb/C SvJ background of the parental floxed PTEN mice. We additionally investigated Balb/C-derived D2OR breast cancer cells as a model of dormancy responsive to environmental cues triggered by PTEN loss in nearby cells, and also examined other, more aggressive syngeneic breast cancer cell lines (D2A, 4T1) for their ability to package Cre into exosomes in our system.
The envisioned approach is shown in Figures 1 and 2 below:

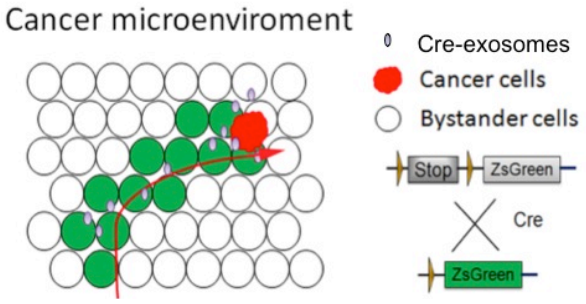


Figure 1: Goal: Exosomal Cre from cancer cells activates expression of green fluorescence in cells that encounter exosomes from migrating cancer cells.

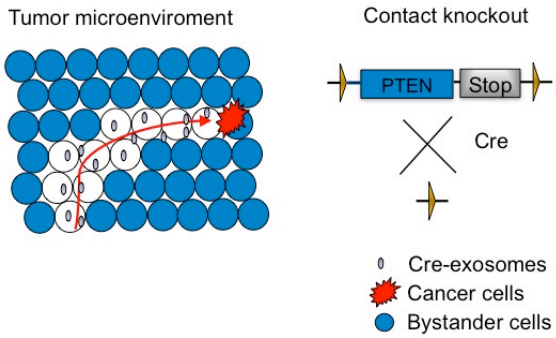


Figure 2: Goal: Exosomal Cre deletes PTEN in cells taking up exosomes from cancer cells

KEYWORDS
Exosome
Phosphatase and tensin homolog (PTEN)
Cre recombinase
Dormancy
Breast cancer

To accomplish these goals, we delineated 5 tasks:
Task 1. Generate cancer cells that secrete a form of Cre-recombinase that can enter and trigger recombination in nearby bystander cells.
Task 2. Prepare mice for study, i.e. generate mice that both delete PTEN and fluoresce green in response to Cre-recombinase
Task 3: Determine function of exosomal Cre-expressing cancer cells in primary tumor model
Task 4. Use exosomal Cre model to determine the effect of PTEN on breast cancer cell dormancy
Task 5. Measurement of the transcriptome of host cells that had contacted cancer cells compared to host cells that had not contacted cancer cells.

We have succeeded in the completion of many of these tasks. The scientific rationale, representative experimental results and conclusions are shared for each task below. A caveat is that in this final year we noted a contaminating cell fusion event that we are analyzing for its impact on *in vivo* results.

Task 1. Generate cancer cells that secrete a form of Cre-recombinase that can enter and trigger recombination in nearby bystander cells.

Background:
Prior to this study, attempts to mediate the cell-to-cell transfer of Cre-recombinase by fusing Cre to protein transduction domains (PTDs) or signal sequences (SS) or a PTD/SS combination had not succeeded. While the fusion proteins could enter cells when prepared as recombinant proteins, cell-directed expression of the Cre-fusions could not mediate effective cell-cell transfer of Cre (without chloroquine or transfection reagent) [2]. In other studies, bacterial or toxoplasma protein-Cre fusion enabled Cre uptake by host cells but required a microbial-directed invasion apparatus [3,4]. We sought to establish exosomal packaging as new, effective delivery mechanism for Cre recombinase into neighboring cells. Exosomes are 40-100nm vesicles that occur naturally and are excreted both by cancer and normal cells.

Results

Cre-recombinase fusions with a fluorescent protein (mKate) and with full length cDNAs from flotillin, mfge8 and CD63 that encode exosomal proteins were generated as this study was about to commence. Of these, the highest exosomal expression was of the Cre-CD63 construct expressed as the fusion: Cre- mKate-CD63. Cancer cells expressing this construct packaged it into exosomes that were transferred to co-cultured green reporter fibroblasts and converted them to green fluorescence as shown in Figure 3:

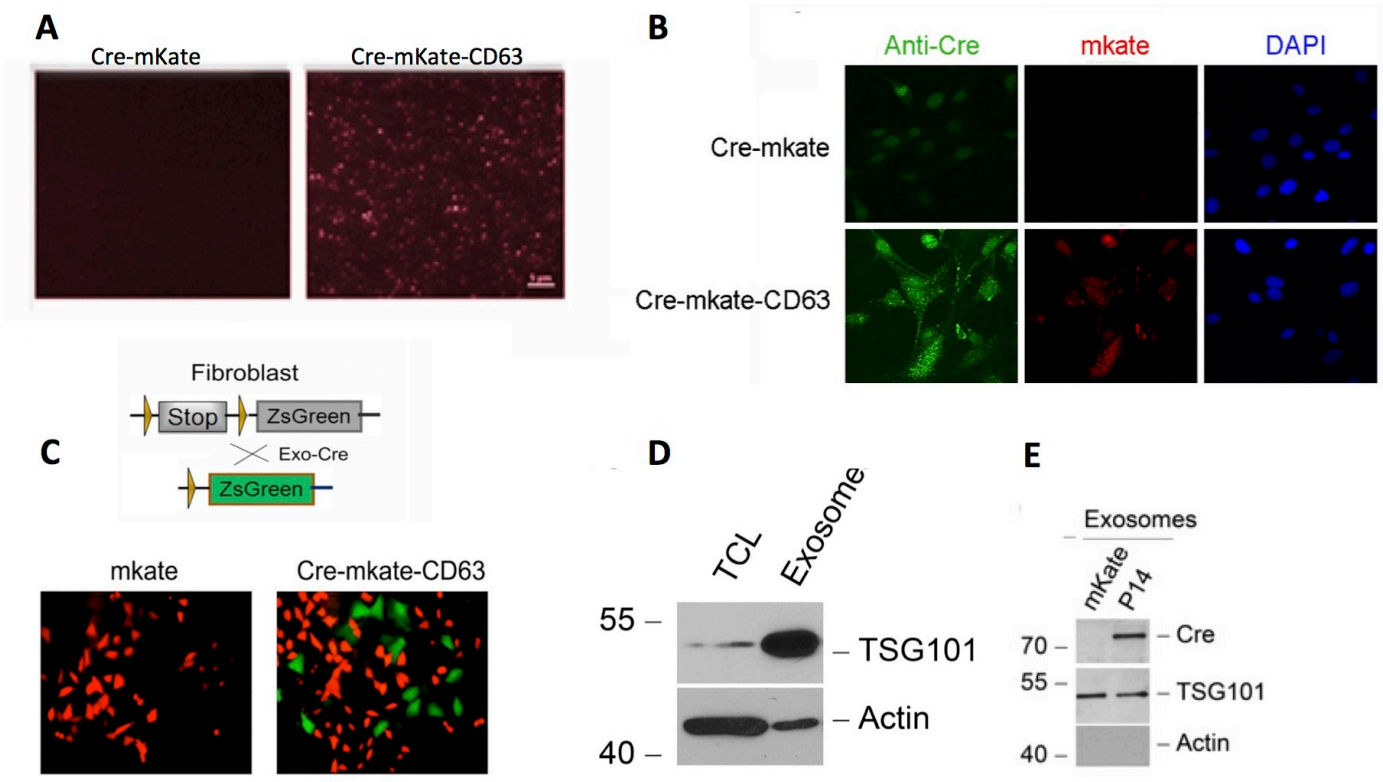


Figure 3: A. Red fluorescent exosomes generated by D2OR cells expressing Cre-recombinase fused with mKate fluor and CD63. No fluorescence of exosomes from Cre-mKate fusions without the targeting sequence. Scale bar 1 um. B. Uptake by fibroblasts of Cre-recombinase in exosomes from cancer cells expressing Cre-mKate-CD63, but not in exosomes from cancer cells expressing Cre-mKate. Staining for Cre (green) is done 1 hour after exosome uptake (before conversion of reporter cells to green). Red exosome fluorescence is only evident in cells taking up the exosomally packaged Cre-mKate-CD63. C. D2OR breast cancer cells (red) expressing the fluorescent tag mKate or expressing mKate tagged Cre-recombinase fused with CD63 were co-cultured 1:1 with ZsGreen reporter fibroblasts for 48 hours; conversion of reporter cells exposed to the exosomal-packaging fused Cre is evident. D. Exosome production by D2OR cells. TCL refers to total cell lysate of D2.OR cells. Exosomes were prepared by differential centrifugation of cells cultured in DMEM containing exosome-free serum. Conditioned medium was centrifuges at 2,000 rpm to remove cells; 20,000 rpm of supernatant to remove debris; filter of that supernatant through 0.22 micron filter, 100,000 rpm centrifugation of filtrate for 2 hours to pellet exosomes. Enrichment for TSG101 is evident. E. Packaging of Cre into exosomes in D2.OR breast cancer cells. A Cre-producing fusion protein (P14, Cre-TSG101, see below) was detected in D2OR exosomes using antibody against Cre.

Electron microscopy and detergent studies suggested that this construct positioned Cre on the outside of the exosome. We also generated (with other support) Cre in an exosome-targeting sequence such that Cre was packaged at high efficiency and maintained inside the exosome The Gould laboratory had reported [5]that HIV gag and plasma membrane proteins were more efficiently packaged into exosomes if they oligomerized into multimers. One approach that they used was to fuse the protein of interest to a synthetic leucine zipper tagged with the fluorescent protein dsRed1 (that itself forms multimers). We experimented with this approach and made a construct comprised of these moieties in the listed order: an Acyl group (for membrane binding)-GFLG (enabling cleavage in the lysosome)-Cre Recombinase-GFLG-Leucine Zipper sequence (from the TSG101 protein for multimerization)-DsRed1 protein (for multimerization). Analysis during task 1 indicated that Cre-recombinase appeared inside the exosome when expressed and packaged from this construct (referred to as TSG101 Cre in Figure 4).

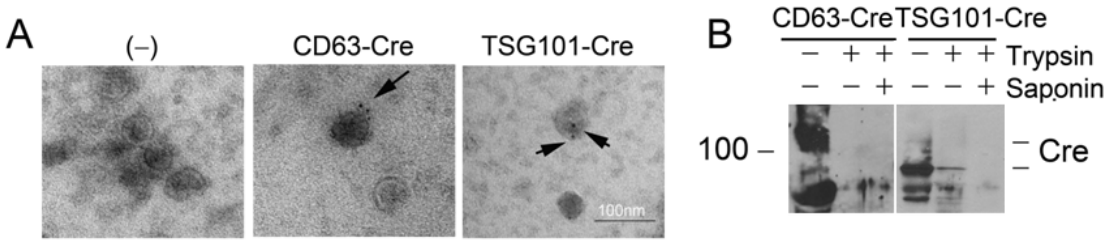


Figure 4. **Differential localization of targeted Cre within melanoma exosomes** (A) Immunogold staining of exosomes from nontransduced cancer cells (-) or cells stably transduced with the Cre-CD63 or TSG101-Cre shows Cre immunoreactivity of transduced CD63-Cre on the outside of exosomes, whereas TSG101-Cre was not clearly outside. (B) Western blot analysis showed complete digestion of CD63-Cre with trypsin independent of saponin treatment. Tryspin treatment resulted in a reduction in Cre signal in TSG101-Cre exosomes, but only the combination of trypsin and (permeabilizing) saponin led to a complete digestion of TSG101-Cre.

Either CD63 or the TSG101 LZ fusion was required for exosomal packaging (Figure 5):

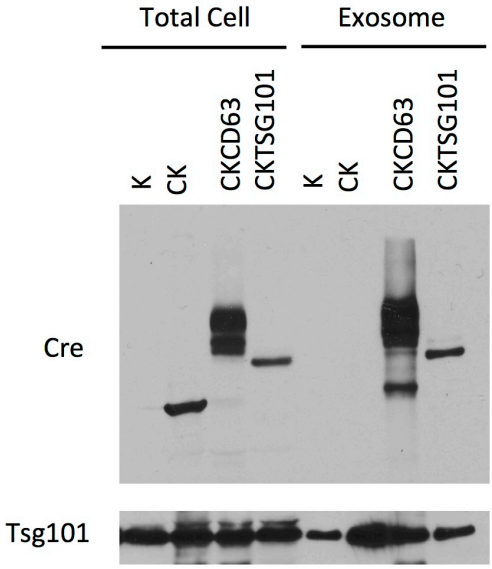


Figure 5, cancer cells lentivirally transduced to stably express either mKate fluorescent protein (K), a fusion of Cre and mKate (CK), CK N-terminal fusion with CD63 (CKCD63) or CK flanked by an Acyl group and the TSG101 LZ sequence/dsRed (CKTSG101). Oncly the CKCD63 and CKTsg101 package Cre into exosomes.

The measure of exosomal Cre transfer and function that we used was conversion of Cre-responsive reporter cells during co-culture with exosomal Cre expressors. Our conversion efficiency was about 5-15% and we sought a higher efficiency for in vivo studies. We determined that chloroquine addition increased conversion of nearby cells (Figure 6), a finding compatible with sequestration of transferred exosomes and Cre in lysosomes and loss of activity following Cre uptake.

We therefore chose to deliver Cre in cis with the following lysosomal disruption peptides [6]:

peptide H5WYG: GLFHAIAHFIHGGWHGLIHGWYG
peptide HA2E5: GLFEAIAEFIENGWEGLIEGWYG
peptide HA2: GLFGAIAGFIENGWEGMIDG
peptide INF7: GLFEAIEGFIENGWEG MIDGWYG
peptide GALA: WEAALAEALAEALAEHLAEALAEALEALAA

The rationale was that these peptides, that are activated at late endosomal or lysosomal pH's would free the fused Cre recombinase from the late endosome or lysosome increasing its nuclear translocation and efficient genetic conversion of the recipient cell. We additionally flanked Cre with lysosomal cleavage peptides to free it from fusion partners (P56 below). Exosomal production and uptake of Cre- into fibroblasts was not impeded by the presence of lysosomal disrupting peptides.

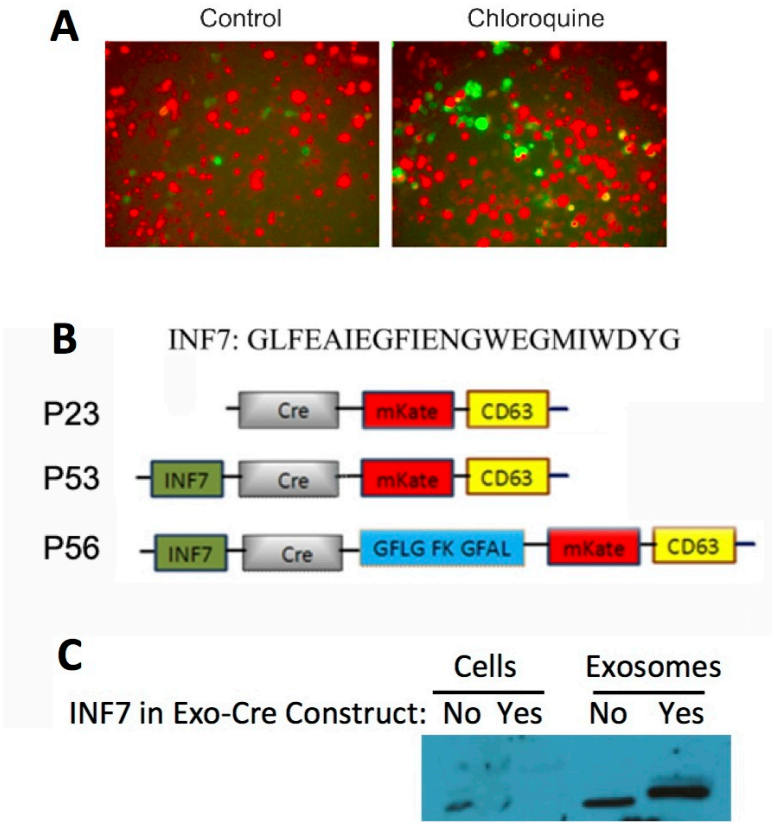


Figure 6. A. The presence of chloroquine increased reporter cell conversion during co-culture of (red) exosomal Cre-CD63 (P23)-expressing cancer cells with reporter ZsGreen fibroblasts. B. CD63 constructs containing lysosomal cleavage and/or disruption peptides. C. Cre Western Blot. Exosomally-targeted fusion proteins are packaged into exosomes despite fusion with a lysosomal disruption peptide.

Similar fusion proteins were made with the TSG101-based exosomal Cre construct (Figure 6D below, constructs B-F). Construct A has been shown to have strong Cre-recombinase activity when co-transfected with reporter plasmids into 293T cells and to be packaged into exosomes. Construct B-F not only retain the ability to be packaged into exosomes, but also have intact Cre-activity when co-transfected with Cre-reporter plasmids. However, conversion of reporter cells by cancer cells expressing a lysosomal escape tethered Cre was not augmented, indicating a distinct rate limiting step.

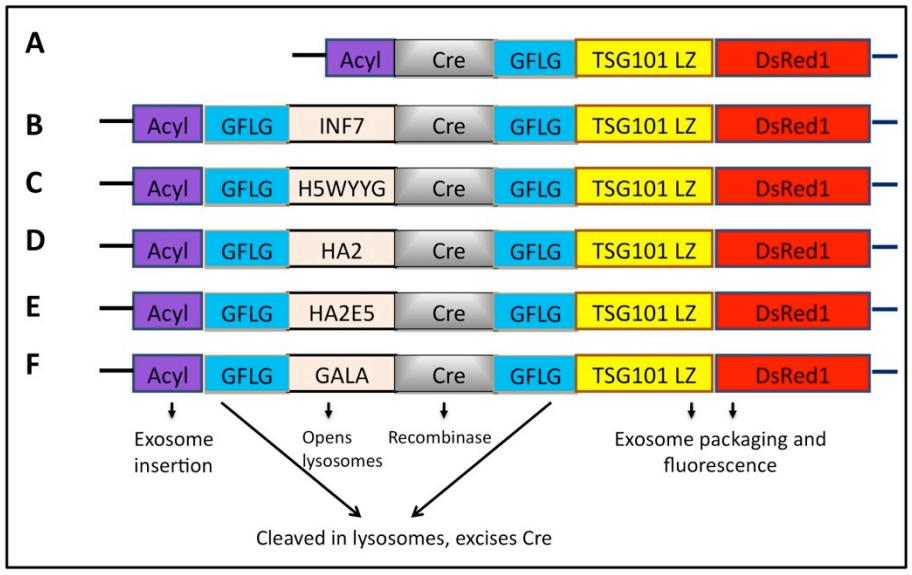


Figure 6D—optimization of TSG constructs.

Over time we noted dropoff of Cre-expression in cancer cells lentivirally transfected with either construct A or B above (with Cre targeted with acyl- and TSG101 peptides), or with Cre tethered to mkate-CD63 or to the mkate-fluorescent tag alone. In order to minimize negative selection associated with Cre- and to benefit from the option of temporal control of exosomal Cre transfer, additional modifications were made to enable the generation of exosomal Cre- by cells to be Doxycycline-inducible. The doxycycline inducibility also enabled extremely high expression of Cre-exosomes under temporal control. This is shown in Figure 7B. We

successfully constructed and generated lentivirus using doxycycline-inducible promoters to drive our exosomal-Cre and control protein production. Figure 7 shows doxycycline inducibility of lentivirally transduced cells—the subpopulation with the highest level of inducibility in a mass culture was subcloned as single cells and expanded without doxycycline. Each of these subclones was expanded and compared for mKate inducibility in the presence and absence of doxycycline and exosomal Cre production was determined under these conditions. While this was an intensive and time-consuming effort it led us to optimal D2.OR sublines with inducible exosomal-Cre or control activity.

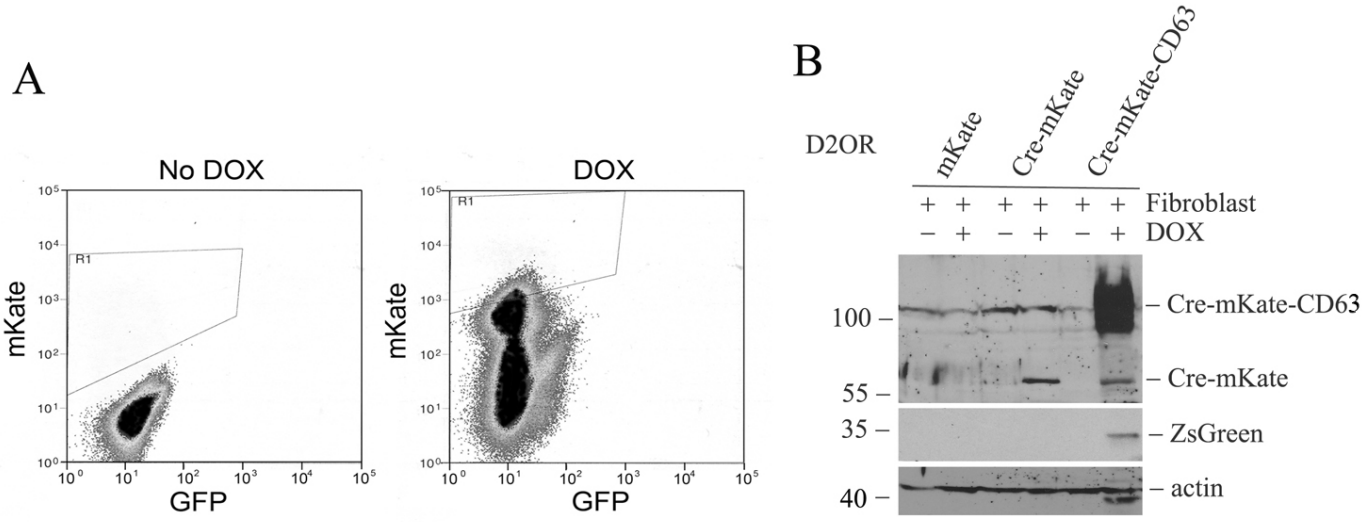


Figure 7. Establishing a Tet-on inducible exosomal Cre producing cell line. (A) FACS sorting regions used for preparation of the D2.OR cells with the most robust expression of fusion proteins. D2.OR cells were transduced with different Tet-on lentiviral constructs and selected with puromycin. Cells were treated with 500ng/ml doxycycline (DOX) for 2 days before sorting. (B) Western blot shows the mKate-Cre-CD63 protein induction in D2.OR cells and induction of ZsGreen after DOX treatment of co-cultured D20R cells tet-inducible for indicated constructs (Western blot shown for Cre, ZsGreen and actin).

Discussion, Task 1

Our work in Task 1 demonstrated the success of our strategy to exosomally package Cre and to enable functional cell-cell transfer of exosomal Cre to effect genetic recombination. We increased the potential of this technology beyond initial plans by enabling doxycycline control of the exosomal Cre expression and transfer system. Although steps designed to augment nuclear Cre-transfer through lysosomal trafficking modulators were not beneficial, exosome uptake consistent induction of genetic recombination (manifested in green fluorescence) in reporter cells was observed.

While these studies were in progress, Zomer et. al. published a report in Cell [7] that demonstrated the ability *in vivo* of aggressive breast cancer cells to send extra-cellular vesicles to non-aggressive cancer cells elsewhere in the mouse and increase the oncogenicity of the latter. Similar to our project, the aggressive cells were transduced with Cre and the targeted cells contained a fluorescent Cre-reporter GFP. The Cre mRNA (but no Cre-protein) trafficked into the extra-cellular vesicles. Because vesicle uptake was accompanied by fluorescent conversion, that suggests that Cre mRNA alone suffices to mediate trans-cellular genomic recombination. We have tested our exosomes and shown that they contain Cre mRNA; however, in contrast with the above findings, we observe little conversion of host cells by exosomes (eg Cre-Kate) containing Cre mRNA but no protein. We have highly overexpressed Cre-Kate in 293T cells and co-cultured them with 293T cells transfected with a Cre-inducible green fluorescent construct. Under these

conditions, in which Cre protein does not traffic to the exosome, we did see modest conversion of the co-cultured 293 reporter cells; however, the efficiency of conversion **was 4-fold less than** that mediated by 293 cells expressing exosomal Cre-protein.

Task 2. Prepare mice for study, i.e. generate mice that both delete PTEN and fluoresce green in response to Cre-recombinase

Background

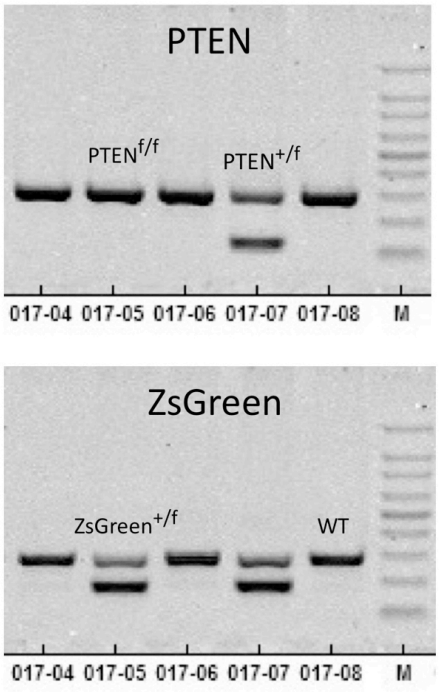
Trimboli et. al. and Wallace et. al. have reported that the genetic inactivation of PTEN in stromal fibroblasts in ErbB2 and in Myc- murine breast cancer models accelerated the onset and progression of mammary tumors. The loss of PTEN was associated with loss of stromal microRNA miR-320 and an increase in the ETS2 transcription factor that resulted in the elaboration of a protumorigenic secretome from the stroma and a buildup of extracellular matrix [8]. This secretome was validated as a prognostic factor in human breast cancer. Collagen 1 was a prominent component of the induced ECM. These papers demonstrated increased cancer vascularization and cancer cell migration arising from PTEN loss in the microenvironment. These studies were performed in oncogene induced cancer models.

Whether the loss of PTEN was capable of activating the growth of nonaggressive or dormant cancer cells was not explored. However, Barkan et. al. studied breast cancer dormancy using the well characterized Balb/C breast cancer cell line D2.OR [9]. In syngeneic transplantation models, these cancer cells disseminate and survive, but remain quiescent as validated using single cell whole organ microscopy. Barkan et. al. found that a fibrotic microenvironment generated using intratracheal adeno-TGFbeta activated the replication of lodged D2.OR cells. In particular, collagen 1 triggered the dormancy to proliferation switch. These findings provide a rationale for the hypothesis that the deletion of PTEN (and resultant upregulation of matrix) in cells in the cancer microenvironment could trigger the exit of D2.02 cells from the dormant state.

A critical step in testing that hypothesis was the generation of a mouse that deleted PTEN in which the proximity of PTEN deleted cells (via induced green fluorescence) to (red fluorescent) D2OR cells could be measured.

Results

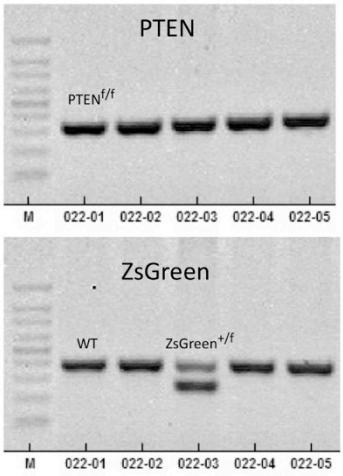
Prior to the onset of this project, we obtained approval from our IACUC proposal entitled Real-Time Visualization and Manipulation of the Metastatic Trajectory of Breast Cancer Cells (protocol #13031479) was approved by the University of Pittsburgh IACUC committee 3/4/13. The first annual renewal was approved on 1/17/14 and we maintained communication with and approval from ACURO throughout the duration of the project.



A breeding pair consisting of a female floxed PTEN BALB/c mouse (JAX 004597 C;129S4-*Ptentm1Hwu*/J) and a male ZsGreen Cre-reporter C57Bl/6 mouse (JAX 007906 B6.Cg-*Gt(ROSA)26Sortm6(CAG-ZsGreen1)Hze*/J) were obtained and bred.

To generate experimental mice required for these studies, the original homozygous floxed PTEN BALB/c female ($PTEN^{f/f}$) was bred with a first generation heterozygous floxed PTEN ZsGreen Cre-reporter male ($PTEN^{+/f}$ $ZsGreen^{+/f}$). As expected, genotyping revealed that this cross produced mice both heterozygous and homozygous for the floxed PTEN allele and heterozygous for ZsGreen (Figure 8, 9).

Figure 8: Confirmation of generation of a female mouse that was both homozygous for floxed PTEN and carrying Cre-inducible ZsGreen reporter transgene (see 017-05) as well as reporter mice heterozygous for floxed PTEN (for example mouse 017-07) as a result of cross breeding protocol.



Genotyping of a third generation litter showed that all pups were homozygous for the floxed PTEN allele. We proceeded to generate Floxed PTEN/ZsGreen Cre reporter mice, backcrossing the C57Bl/6 ZsGreen reporter mouse x Balb/c f/f-PTEN mouse onto Balb/c background for 7 generations to optimize its use as a host for the (Balb/c) D2.OR breast cancer cells that are known to model breast cancer dormancy.

Mice that were PTEN$^{f/f}$ ZsGreen$^{+/f}$ were bred to double homozygosity to be used for *in vivo* experiments. Although a facility pinworm infection and associated restrictions slowed our progress in this task, we achieved over time an adequate number of double transgenic progeny to proceed.

Figure 9. Example of a male mouse that is homozygous floxed PTEN and carries Cre-inducible ZsGreen

We validated Cre-induction of ZsGreen in progeny by tranfecting fibroblasts generated from a PTEN$^{f/f}$ ZsGreen$^{+/f}$ pup with msCre using the Amaxa/Lonza nucleofector system for primary fibroblasts. Figure 10 shows conversion of transfected fibroblasts to green fluorescence and deletion of PTEN by Cre:

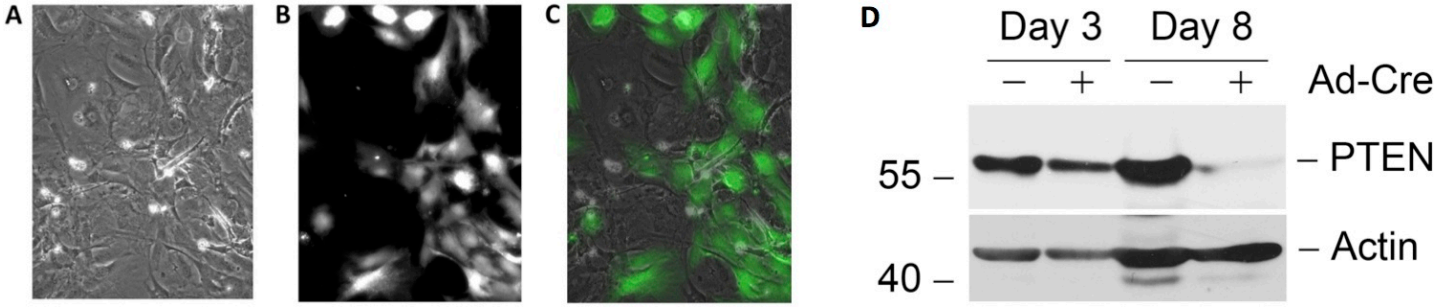


Figure 10. PTEN ZSGREEN mouse fibroblasts transfected with msCRE: A. Brightfield; B. Green fluorescence; C. Brightfield overlaid with Green. D. Deletion of PTEN in double transgenic fibroblasts infected with Cre-expressing adenovirus.

Cre-packaging exosomes from D2OR cells could convert the floxed ZsGreen PTEN fibroblasts to green fluorescence (Figure 11).

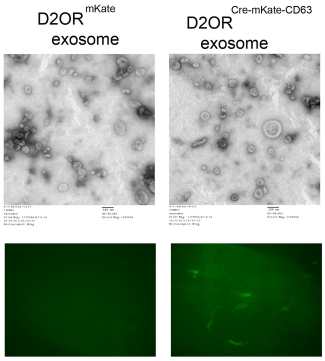


Figure 11. Exosomes isolated from control (D2OR mKATE) or exosomal Cre- producing (D20R Cre-mKate-CD63) cells were characterized by TEM (above) and for their ability to convert ZsGreen reporter fibroblasts to green fluorescence (below).

Discussion, Task 2

The double transgenic mouse (f-stop-f-ZsGreen$^{+/+}$/f-PTEN-f$^{+/+}$) generated to homozygosity is a resource to

enable visualization of PTEN deleted cells that should be of use in PTEN biological studies broadly and enabled us to move forward to test the hypothesis that cancer cell dormancy relies on PTEN expression in nearby noncancerous cells in the microenvironment. The fibroblast cell lines derived from this model should also be a resource both *in vitro* or in *in vivo* cell transfer models. We were able to show that the fibroblast cell line served as a fluorescent reporter of the action of isolated or inter-cellularly-transferred exosomes containing Cre-recombinase fusion proteins.

Task 3: Determine function of exosomal Cre-expressing cancer cells in primary tumor model.

Background

Based on analysis of PTEN-knockout mammary fibroblasts, the deletion of PTEN leads to secretion of multiple proteins that are liberated from suppression downstream of the miR-320 microRNA [8]. Given that expression of this secretome in clinical samples was correlated with breast cancer recurrence, it could represent a paracrine promoter of breast cancer growth or exit from dormancy. Loss of stromal PTEN has also been linked to collagen secretion [1]. Collagen has been shown to trigger growth of D2OR breast cancer cells in the lung, where they otherwise exist in a dormant state [9,10]. Having set up the mouse model Cre-mediated fluorescence and PTEN loss in Task 2, and enabled D2OR cells to express transferrable Cre-recombinase in Task 1, we now sought to study this interaction of these cells *in vitro* and *ex vivo.*

Results

Prior to undertaking *in vivo* experiments, we wanted to establish the kinetics of stromal cell genetic recombination in the presence of D20R cells transferring Cre-recombinase through exosomes. We set up a live cell imaging kinetic study to characterize how long after doxycycline addition the cancer cells began to express the (red fluorescent) Cre-fusion protein, and when in relation to this we first observed green fluorescence of adjacent cells. As shown in Figure 12, this analysis indicated that approximately 5 days should elapse for induced cancer cells to genetically remodel adjacent tissue at loxP sites, and informed our planning for *in vivo* sampling.

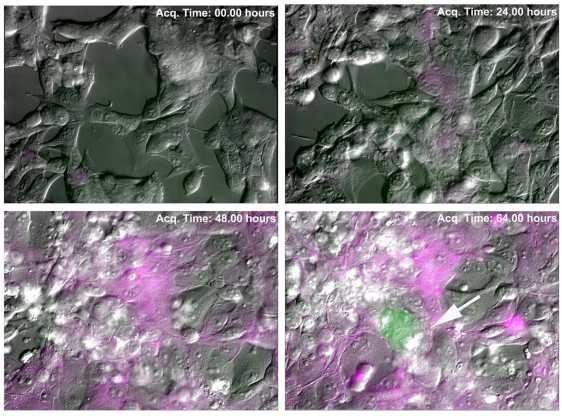


Figure 12. Time course (0-64 hours, extracted from live cell video) of doxycycline induction of Cre-mKate-CD63 exosomes in D20R cancer cells (red) and resultant exosomal transfer to reporter fibroblasts with resultant ZsGreen expression.

In addition to ZsGreen induction, we needed to show concurrent deletion of PTEN in fibroblasts of double transgenic mice exposed to cancer cell Cre exosomes. This is demonstrated in Figure 13, in which the absence of PTEN in the ZsGreen-converted cell is evident:

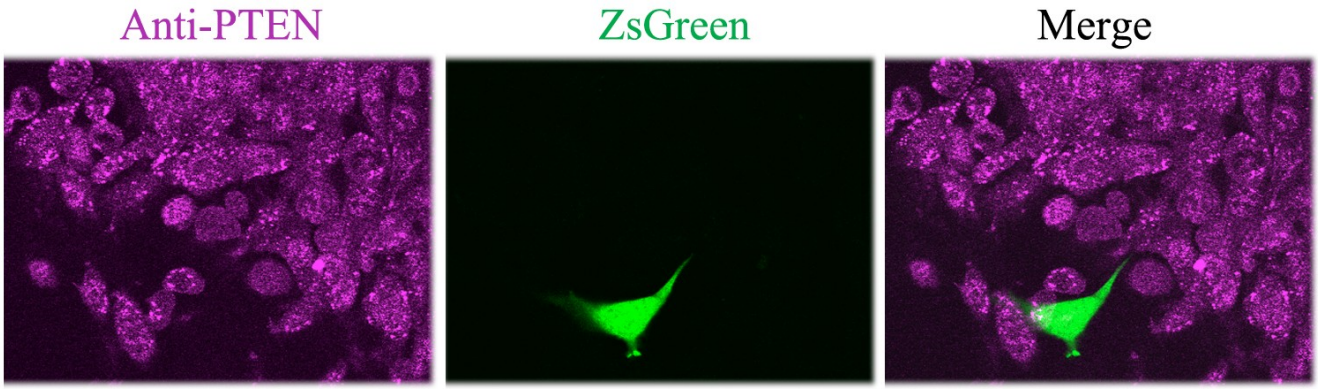


Figure 13. Induction of ZsGreen is concurrent with loss of PTEN in double transgenic fibroblasts. PTEN staining is red, ZsGreen fluorescence green.

Prior to proceeding in animals, we set up matrigel cultures to confirm low levels of D2OR growth and then determine whether D2OR growth could be stimulated by paracrine factors elaborated by PTEN deleted fibroblasts. Notably, in 2-dimensional cultures D2OR cells grow robustly, comparable to the growth rate of the parental D2A1 breast cancer clone that (in contrast to D2OR) is aggressive *in vivo*. Figure 14 demonstrates nonaggressive growth of the D2.OR cell line as compared with its parental D2.A1 cell line in 3D culture.

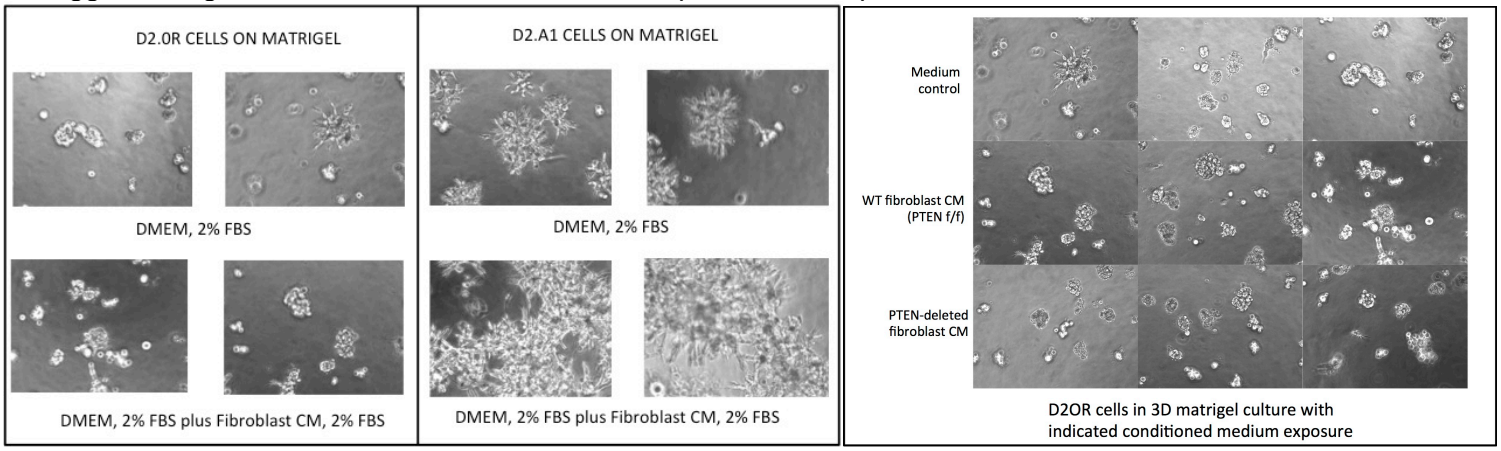


Figure 14: Left. Nonaggressive growth of the D2.OR cell line as compared with its parental D2.A1 cell line. 3D culture for 9 days in matrigel with or without added wild type (PTEN f/f) fibroblast conditioned medium (CM) is shown. D2.OR is a murine breast cancer cell line for syngeneic transplantation into Balb/C mice. Right. Conditioned medium from PTEN-deleted fibroblasts did not augment D20R growth.

Our observation of non-augmented D2OR growth from PTEN-deleted medium did raise the possibility that growth of this cell line would not be bolstered by Cre-mediated deletion of microenvironment PTEN. However, we felt that an *in vivo* test was warranted given that a combination of juxtacrine and paracrine crosstalk could be productive or that augmentation of metastasis from subcutaneous or mammary fat pad injection could also follow microenvironment PTEN deletion.

We commenced experiments to determine if Cre-exosome expression and resulting host stromal recombination enabled D2.OR cells to escape dormancy. We injected mammary fat pad of Cre reporter ($PTEN^{f/f}$ $ZsGreen^{f/f}$) mice with 1 million D2OR cells expressing doxy-inducible mKATE or Cre-mKate-CD63 and exposed them to drinking water with or without 200mg/ml doxycycline. Mice were sacrificed on day 11 and tumor removed and fixed tissue stained with DAPI (nuclear stain) and with anti-ZsGreen (1:100) and Alexa-488 secondary. mKate was directly visualized. Results as shown in Figure 15 indicated doxycycline associated expression of mKate and conversion of adjacent cells to green fluorescence for the Cre-mKate-CD63

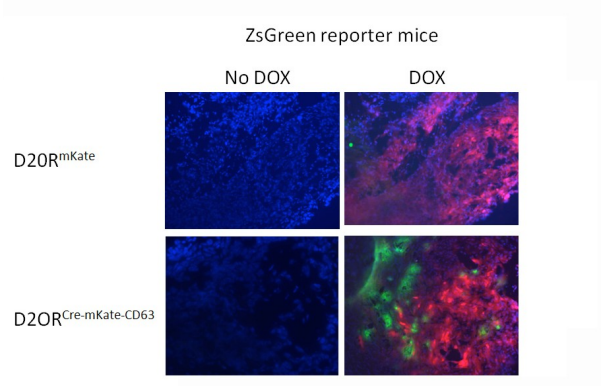


Figure 15. Mammary fat pad injection of $PTEN^{f/f}$ $ZsGreen^{f/f}$ mice with D2.OR cells stably-expressing indicated doxycycline-inducible constructs. Merged images of DAPI (nuclei, blue), ZsGreen Alexa-488 immunostaining and mKate (red) immunofluorescence.

However, on attempts to duplicate these findings, we were unable to derive palpable D20R tumors either with mammary or subcutaneous injections up to 45 days from the point of injection. Tumor was undetectable upon dissection whether or not exosomal Cre was induced by doxycycline, suggesting that the activation of these cells from a dormant state could require additional factors beyond a PTEN-deleted host environment. Because D2.OR did not grow out under these conditions, we extended our

study, as per decision point outlined in our original plan (subtask 3C), to other Balb/C syngeneic cancer cell lines, 4T1 and D2.A1. These lines were rendered capable of exosomal Cre production and transfer under doxycycline control (Figure 16).

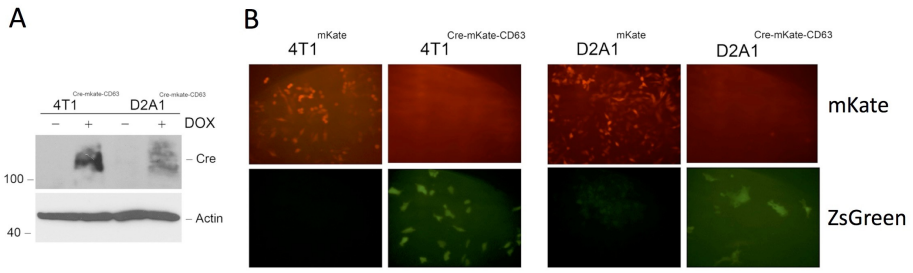


Figure 16. A. Induction of Cre-fusion protein by doxycycline in stably lentivirally-transduced clones of 4T1 and D2A1 breast cancer cells. B. Demonstration of functionality of 4T1 and D2A1 exosomal Cre in converting co-cultured reporter fibroblasts.

Discussion, Task 3

While our results suggested at the time that cell-cell transfer of Cre-packaged in exosomes could proceed *in* vivo, we chose based on both our *in vitro* (matrigel) and *in vivo* (inadequate primary tumor for analysis) to defer studies on D2OR dormancy and to focus instead on cancer/microenvironment modulation by more aggressive syngeneic cancer cell lines and whether induced expression of Cre fusion proteins in their exosomes could alter cancer cell growth or metastasis.

Task 4. Use exosomal Cre model to determine the effect of PTEN on breast cancer cell dormancy

Background

In modifying Tasks 4 and 5 because of challenges with the dormancy model, we still focused on the *PTEN$^{f/f}$ ZsGreen$^{f/f}$* as an ideal model to show exosomal Cre conversion of host tissue. The focus shifted to study of whether PTEN deletion could be effected by more aggressive (4T1, D2A1) breast cancer cells transferring Cre and whether this genetic deletion in the host was protumorigenic.

Results

We characterized the PTEN deleted, ZsGreen-positive fibroblasts for signals associated with loss of the PTEN phosphatase and for the induction of potentially oncogenic mRNAs and proteins. As shown in Figure 17, loss of PTEN was associated with activation of downstream kinases known to be suppressed by PTEN tumorigenicity (Figure 17). We also analyzed f/fPTEN-f/s/fZsgreen fibroblasts before and after Cre-mediated recombination in directed measurement of paracrine signals that could arise in the altered stroma to bolster cancer growth. As shown in Figure 17B the PTEN-deleted fibroblasts upregulated MMP9 and alpha- smooth muscle actin transcripts. MMP9 is pro-angiogenic and has been associated with increased 4T1 migration(*3*) and lung metastases(*4*).

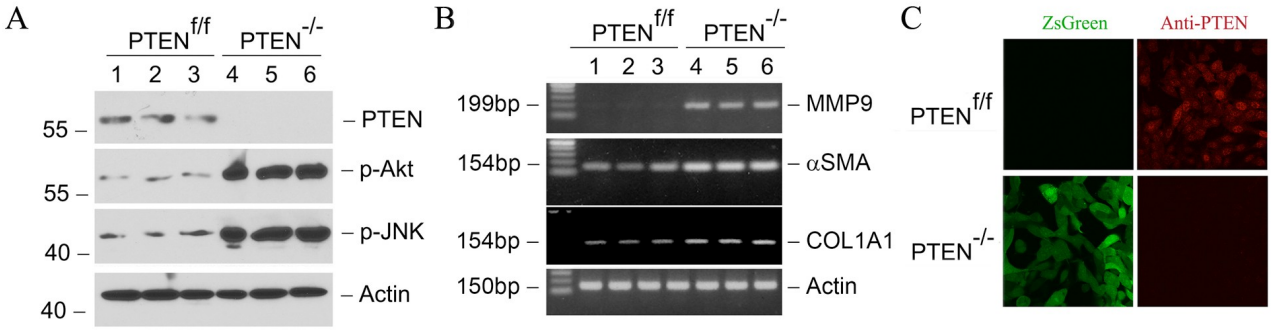


Figure 17. Effect of PTEN deletion on kinase activation and transcripts of select secreted proteins. A. activation of p-AKT and p-JNK by PTEN deletion; 3 independent determinations shown; B. Upregulation of MMP9, αSMA and collagen transcripts; C. Activation of ZsGreen and loss of PTEN immunoreactivity

in Cre-treated fibroblasts.

Before testing 4T1 and D2A1 cells *in vivo* we determined whether conditioned medium from PTEN-deleted (versus PTEN-floxed) fibroblasts increased 4T1 growth in matrigel. As shown in Figure 18, paracrine factors from the deleted fibroblasts promoted growth and a mesenchymal phenotype of 4T1. These results were compatible with the high level of c-myc expression in 4T1 [11] an oncogene that has been linked to an exuberant cancer cell response to PTEN-deleted fibroblasts [12].

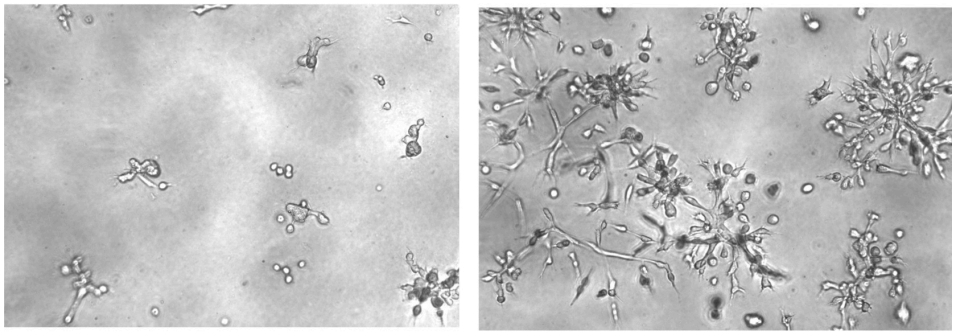
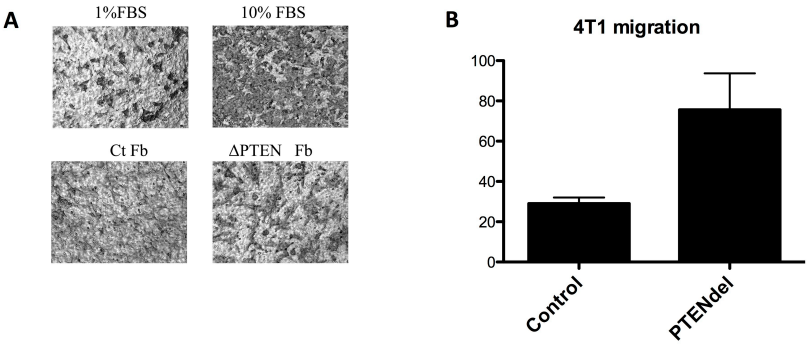
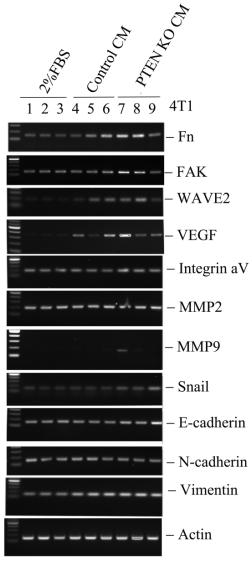


Figure 18. 3D culture of 4T1 cells exposed to conditioned medium from wild type or PTEN-deleted fibroblasts.



Additionally, PTEN-deleted fibroblast CM increased migration of 4T1 cells *in vitro* (Figure 19).

Figure 19. Increased 4T1 migration by CM of PTEN-deleted fibroblasts. A. Photographs from representative transwell assay; Ct=control, Fb=fibroblast. B. Quantitation of migration experiments, p=0.011.



PTEN conditioned medium also affected gene expression by the 4T1 cells, with increased expression of the EMT-associated transcript *Snail* although no alteration in N- or E- cadherin evident in RT-PCR (Figure 20).

Figure 20. 4T1 breast cancer cells exposed for 72 hours to low serum with or without conditioned medium from wt (floxed) control fibroblasts (Control CM) or from the PTEN-deleted fibroblasts. Triplicate samples shown for each condition.

Given these *in vitro* results, we expected that if the exosomal-Cre-expressing 4T1 model caused peritumoral PTEN-deletion *in vivo,* that it would enhance tumorigenicity. Because 4T1 cells did not express Cre in the absence of doxycycline, the experiments would compare 4T1 growth and spread (Ki-67, tumor growth, lung metastases) in the presence and absence of doxycycline. We also planned to

use immunofluorescent staining to measure microenvironment protein changes induced by the cancer exosomes (including stromal MMP9 and secreted smooth muscle actin) and the proximity over time of cancer cells to stromal cells that lose PTEN expression (Task 5).

We pretreated exosomal-Cre generating 4T1 cells (CreKate-CD63) with or without doxycycline for 3 days, followed by intravenous injection into mammary fat pad (1 million cells, 2 experiments), subcutaneous injection (200,000-1 million cells, 3 experiments) or intravenous injection (500,000 cells, 2 experiments). 4T1 cells expressing mKate were injected into a subset of mice as an additional control. Surprisingly, despite the Balb/C-SvJ background of our PTEN$^{f/f}$ ZsGreen$^{f/f}$ mice, growth of the syngeneic 4T1 cells (either our transduced cells or parental non-manipulated cells) was not robust and tumor rapidly regressed to a nonpalpable state in two of the subcutaneous and one of the mammary fat pad experiments.

We were unable to detect mKate positive tumor in lung from the mammary fat pad or subcutaneous injections. From intravenous injection, we harvested lung 14 days after IV injection of 500,000 4T1 cells pre-treated for 3 days with doxycycline or vehicle and maintained with or without doxycycline at 200 ug/ml in drinking water. As shown in Figure 21A and B, areas containing both green and red fluorescent cells were detectable dispersed within lung. However, we noted (21B) that some cells expressed high expression of both fluors suggesting exuberant exosomal transfer of mKate, however a red (mKate)-only Cre-expressing cell was not adjacent to the converted cells to account for maintenance of red fluorescent exosomal uptake over this timecourse. We also observed some areas the contained ZsGreen positive, PTEN-negative cells without detectable mKate-expressing cells (21C).

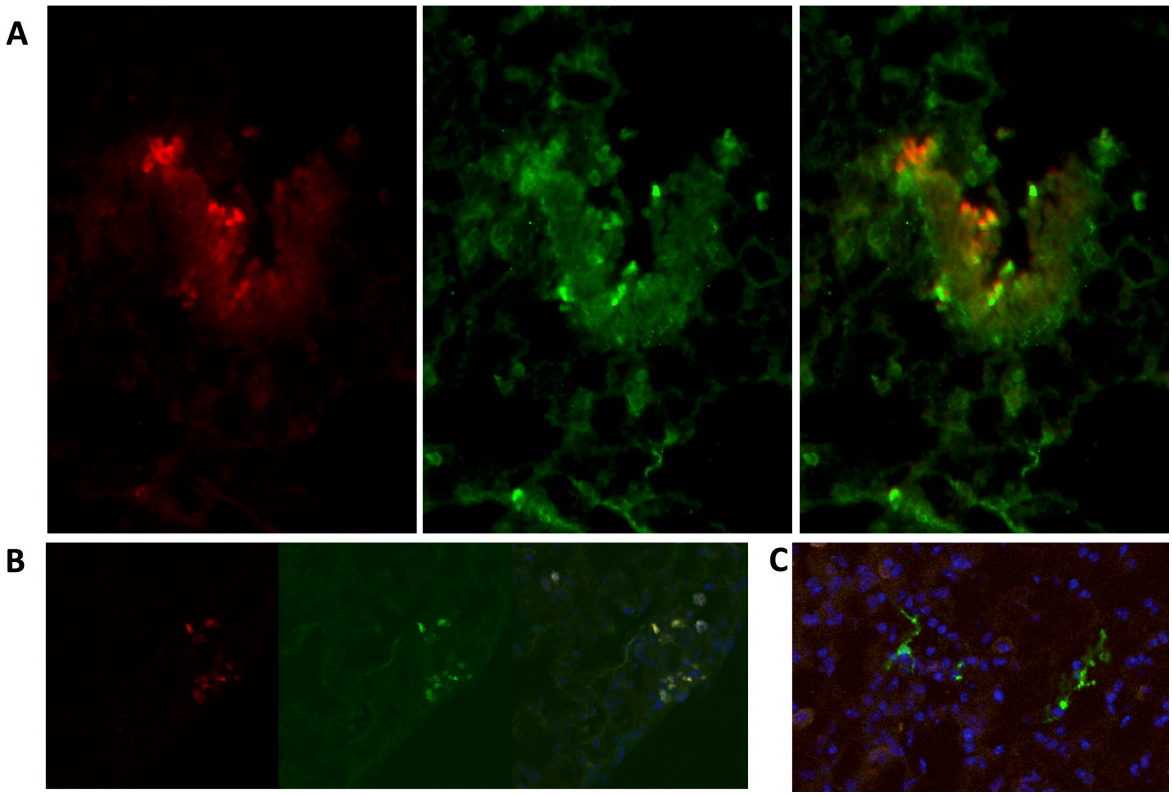


Figure 21. Lung from PTEN$^{f/f}$ ZsGreen$^{f/f}$ mice injected with Cre-Kate-CD63 expressing 4T1 cells and maintained on doxycycline. A. Lung metastasis imaged for mKate (left), ZsGreen (middle), merged image (right). B. Area of dispersed cell fluorescence in which cells are doubly positive for mKate and ZsGreen. C. Merged image of ZsGreen-positive cells (detected with anti-ZsGreen antibody) in area co-labeled with DAPI nuclear label (blue) and immunostained for PTEN (orange). Green converted cells are PTEN-negative, however, no mKate positive cancer cells were visualized in this area. Green immunofluorescence was not detected in tissues of mice not exposed to doxycycline.

Although the above results indicated that 4T1 cells expressed exosomal Cre- upon doxycycline exposure could convert host cells *in vivo*, we could not explain the occurrence of isolated double positive (mKate/ZsGreen) cells observed based on our exosomal transfer paradigm (21B). Staining controls ruled out overlap of fluorescent windows. Before proceeding to collect and analyze converted cells (Task 5) we sought to account for this.

We first investigated whether double positives could arise in co-culture. Flow analysis of one of our D2OR subclones that expressed doxy-inducible exosomes indicated that, as before, following doxycycline addition to exosomal-Cre-induced cancer cells cultured with ZsGreen reporter fibroblasts led to ZsGreen fluorescence. However, pre-staining of the fibroblasts with violet-CSFE indicated that the co-cultured fibroblasts were not the source of green fluorescence, but rather, a subset of the mKate-expressors fluoresced green (Figure 22). We considered that this particular subclone (although it had been sorted 3 times successively for mKate in the presence of doxycycline to isolate a homogeneous high induced expressor of mKate-Cre-CD63) could have been contaminated during passage by (non-CFSE stained) fibroblasts.

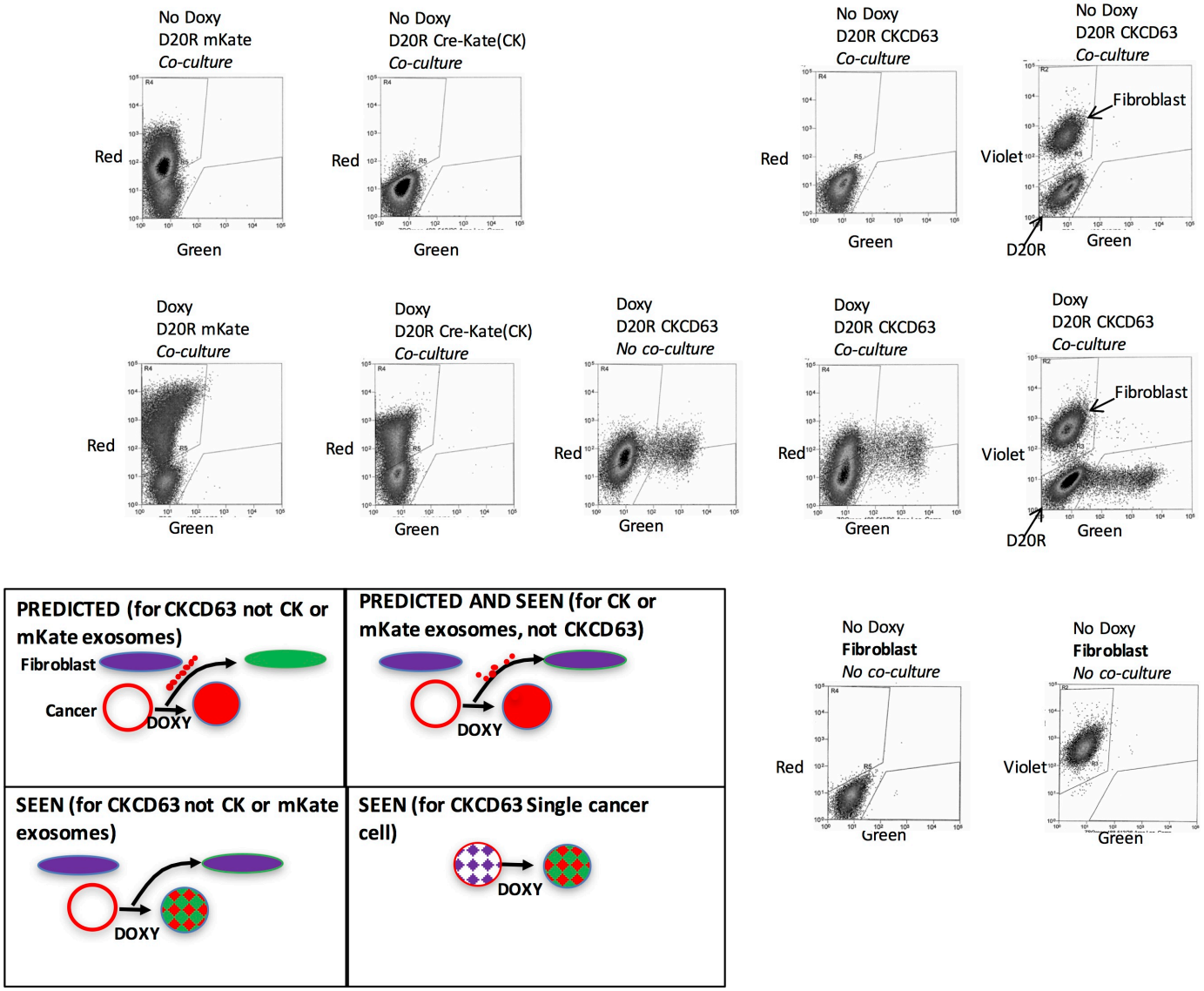


Figure 22. D2OR cells harboring mKate, Cre-Kate, or Cre-Kate-CD63 (CK-CD63) were cultured alone in the presence of Doxycycline or co-cultured with CFSE-violet-stained PTEN$^{f/f}$ ZsGreen$^{f/f}$ reporter fibroblasts. Fibroblasts were also cultured alone. Cells from all cultures were analyzed on flow cytometry for red (doxy-induced mKate), Violet (fibroblast), or green (induced ZsGreen) fluorescence. The presence of double red/green CK-CD63 cells after doxycycline exposure even in the absence of co-culture was evident. Inset shows expected outcomes and those seen, considering also the results of single cell culture (Figure 23).

In order to clarify the flow result, we again cloned and expanded single cells from this D2OR-CK-CD63 subclone. PCR analysis showed that the single cells expressed both Cre recombinase (in accordance with the double lentiviral transduction and dual antibiotic selection generating doxycycline-inducible Cre) but also ZsGreen and the floxed PTEN allele characteristic of the double transgenic fibroblasts (Figure 23). Morphologically, these single cell clonal isolates resembled neither the cancer nor fibroblast lines.

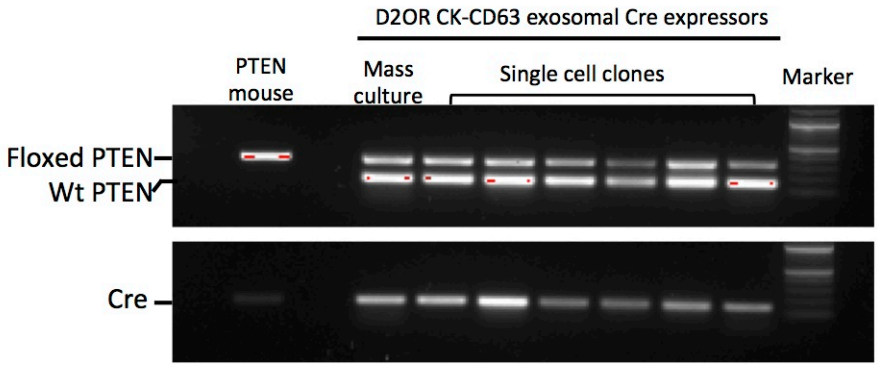


Figure 23. Presence of floxed-PTEN and of Cre- DNA in single cells isolated and grown from D2OR-CK-CD63 subclone. The size of the floxed PTEN band is shown from the PTENf/f/ZsGreen mouse. PCR on the mass culture of the D2OR subclone and on the single cell-derived colonies show the presence of both floxed and wild-type PTEN and of Cre, suggesting that this subline grew out following a cell fusion event.

We suspect that a contamination event arose during the isolation of this high-expressing subclone followed by cell fusion and are determining whether fusion events can occur and whether they are promoted by high expression of CD63. We note that in an HIV model, whereas the tetraspanins CD9 and CD81 oppose viral/host cell fusion, that the expression of CD63 appears to augment fusion [13].

With the possibility that fusion events rather than exosomal transfer, could contribute to a subset of the genetic recombination observations that we see *in vitro* and *in vivo,* we are deferring on drawing firm conclusions from our data until this is further assessed.

4. KEY RESEARCH ACCOMPLISHMENTS

Generation of a dual transgenic floxed PTEN, ZsGreen reporter mouse.

5. CONCLUSION

Our work has demonstrated novel way to package Cre recombinase into exosomes in cells of interest, raising prospect that those cells can alter the genetics of neighboring cells in Cre-responsive mice in a topologically-restricted fashion and under temporal control of doxycycline. Both fusions of CD63- and flanking fusions of an acyl- group and the leucine zipper from TSG101 sufficed for packaging of Cre- into exosomes. Generated exosomes were capable of inducing genetic recombination from floxed sites in cells exposed to them. Co-culture of cancer cells generating exosomal Cre with floxed reporter cells also led to genetic recombination of the reporter cells *in vitro* and *in vivo.* This is presumed to have arisen from transfer of exosomes bearing Cre-recombinase from the cancer cells to the reporter cells. However, we have detected some occurrence of reporter cell conversion that appear to arise from cell fusion, and so the relative contribution of fusion versus exosomal transfer events remains to be established. In the conduct of this work, we generated a dual transgenic floxed PTEN, ZsGreen reporter mouse that should be broadly usable in studies of PTEN biology.

6. PUBLICATIONS, ABSTRACTS AND PRESENTATIONS
Presentation: Women's Cancer Research Center, Magee Women's Hospital, Pittsburgh 5/5/16
Great Lakes Regional Breast Cancer Conference, 9/8/16

7. INVENTIONS, PATENTS AND LICENSES
Nothing to report

8. REPORTABLE OUTCOMES
Nothing to report

9. OTHER ACHIEVEMENTS
Nothing to report

10. REFERENCES

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11. APPENDICES

N/A